Identification of Somatomedin-like Polypeptides Produced by Mammary Tumors of BALB/c Mice


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ABSTRACT

A transplantable mammary tumor derived from an outgrowth of nodule-like alveolar lesions induced by 7,12-dimethylbenz[a]anthracene in cultures of whole mammary gland was shown to produce a family of somatomedin-like polypeptides when cultured in vitro. Minced mammary tumor tissue as well as monolayer cultures of tumor cells produced similar polypeptides when incubated in serum- and hormone-free medium. The polypeptides released into the medium ranged in molecular tides when incubated in serum- and hormone-free medium.

While there is an extensive literature describing the production of hormones and growth-promoting substances by transformed cells, most of this information has come from fibroblasts or sarcomas, and the role of these factors in the in vivo genesis of tumorigenicity remains obscure. In this report, we present the first evidence for the production of a somatomedin-like growth factor by a chemically induced mammary carcinoma.

MATERIALS AND METHODS

Conditioned Medium from Tumor Cells. The serially transplantable mammary carcinoma utilized in these studies was derived from nodule-like alveolar lesions induced by 7,12-dimethylbenz[a]anthracene in cultures of whole mammary organ of female low-mammary-tumor strain BALB/c mice (8, 16). Two different procedures were used to obtain medium conditioned by mammary tumor cells. In the first procedure, small rapidly proliferating mammary tumors were excised, weighed, minced, and washed extensively with serum-free DMEM containing 10 units of penicillin per ml and 100 μg of streptomycin sulfate per ml. The washed tissue fragments were incubated for 6 hr in the same medium at 37° in 5% CO₂:95% air. The medium was then changed, and the incubation was continued for an additional 12 hr in fresh serum-free DMEM under identical conditions. The 12-h conditioned medium was harvested and centrifuged at 10,000 rpm in a SS-34 Sorvall rotor to remove debris and then frozen at −20°.

In the second procedure, minced tumor tissue was trypsinized at 22° for 30 min in a solution of 0.05% trypsin:0.05% ethylene glycol bis(β-aminoethyl ether)-N,N',N''N'''-tetraacetic acid. The cells were harvested by low-speed centrifugation (400 × g; 15 min) and plated at a density of 5 × 10⁶ cells/ml in 10-cm plastic cell culture dishes in DMEM containing 10% calf serum. After 24 hr, the medium was changed to serum-free DMEM. The medium was changed every 48 hr thereafter, and beginning with the third media change, the conditioned medium was harvested, centrifuged at 400 × g for 15 min to remove cell debris, and frozen at −20°. Cells survived extremely well with no decrease in cell number under serum-free conditions, maintained an epithelial-type morphology, and formed characteristic dome structures. The harvest times were chosen after control experiments in which it was demonstrated that activity in the conditioned medium reached a maximum value at the times indicated.

Column Chromatography. Conditioned medium obtained from minced tumors was dialyzed against 2% acetic acid, centrifuged to remove precipitated material, and concentrated by lyophilization. The residue was resuspended in 1 ml acetic acid and chromatographed over a 1 × 120-cm column of Sephadex G-50 resin equilibrated at 4° in 1 ml acetic acid. Fractions of 2.5 ml were collected at a flow rate of 6 to 8 ml/hr using 1 ml acetic acid as eluant. Aliquots (0.25 ml and 0.5 ml)
of the fractions were frozen, lyophilized, resuspended in PBS (Mg²⁺ and Ca²⁺ free, pH 7.4), and assayed for protein content and for ability to stimulate DNA synthesis.

Assessment of DNA Synthesis-stimulating Activity. Aliquots of Sephadex G-50 fractions and tumor cell-conditioned medium were assayed for their ability to stimulate DNA synthesis in quiescent cultures of chick embryo fibroblasts as described previously (7, 13). Briefly, the material to be assayed was added directly to 35-mm cultures of secondary quiescent chick embryo fibroblasts under serum-free conditions. Twelve hr later, the cultures were pulse labeled with [³H]thymidine (0.2 μCi/ml) for 1 hr at 37°. The cultures were washed with PBS and precipitated with 10% trichloroacetic acid at 4°, and incorporation of acid-precipitable radioactivity was determined by liquid scintillation spectrometry.

Competitive Binding Assays. For these studies, we have used MSA as a somatomedin analog. MSA refers to a family of well-characterized polypeptides purified from medium conditioned by the growth of a rat liver cell line (BRL-3A) (4–6, 12). MSA closely resembles the somatomedins and the insulin-like growth factors (IGF I, IGF II, and NSILA-s) in physical and biological properties (9, 12). MSA was purified from serum-free BRL-3A conditioned medium as described previously (7, 9, 12) using preparative scale disc gel polyacrylamide electrophoresis in acetic acid:urea at pH 2.7. Purified MSA consisted of a single species with a molecular weight of approximately 9000 and probably corresponds to MSA polypeptide II-1 in the designation system of Moses et al. (9). MSA was active in stimulating DNA synthesis in chicken embryo fibroblasts at concentrations of 10 to 200 ng/ml.

A partially purified preparation of growth-stimulatory peptides (MTF) from the low-molecular-weight region of the G-50 column was assayed for its ability to compete with [¹²⁵I]-labeled MSA for somatomedin receptors on chick embryo fibroblasts and for the binding of [¹²⁵I]-labeled EGF to EGF receptors on mouse embryo fibroblasts. Quiescent chick embryo fibroblast cultures (2.5 × 10⁶ cells/plate) and mouse embryo fibroblast cultures (2.8 × 10⁶ cells/plate) were prepared in 60-mm plastic cell culture dishes by serum starvation for 48 hr. In both assays, 10⁶ cpm of ligand (¹²⁵I-labeled MSA or ¹²⁵I-labeled EGF) were added to cultures in 1.0 ml of binding medium (DMEM buffered with 0.1 M 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid, pH 7.8, containing 10 mg of bovine serum albumin per ml). Appropriate amounts of the competing peptides, MSA, EGF, or MTF, were added simultaneously from concentrated stock solutions. Binding was allowed to proceed for 2.5 hr at 22° to attain equilibrium. At equilibrium, the cultures were rapidly rinsed 4 times with cold PBS, pH 7.4, and drained, and the cells were lysed with 1.0 ml of hot 1% sodium dodecyl sulfate. Aliquots were assayed for [³H]thymidine incorporation by liquid scintillation spectrometry at 60 to 70% counting efficiency. Iodine-labeled MSA and EGF were purchased from Collaborative Research, Inc. (Waltham, Mass.).

Electrophoresis. Electrophoresis in 4 M acetic acid:8 M urea was performed as described previously (4). Samples to be electrophoresed were lyophilized to dryness and resuspended in 0.05 ml of acetic acid:urea (4 M: 8 M) containing 0.01% methylene blue as tracking dye. The samples were applied to 0.5- x 8.0-cm tube gels of the following composition: 4 M acetic acid;8 M urea; 7.5% acrylamide (w/v); 0.25% methylenebis[acylamide] (w/v); 0.5% N,N',N",N"-tetramethylethelenediamine (v/v), and 0.21% ammonium persulfate (w/v). Electrophoresis was carried out for 4 to 6 hr at 22° toward the cathode. Gels were stained in a solution of 0.05% Coomassie blue (R-250) in methanol:acetic acid:H₂O (5:1:5) for 1 to 2 hr at 37°. The gels were destained in 7.5% acetic acid (v/v) containing 20% methanol.

RESULTS

Assay of Mammary Tumor Cell-conditioned Medium for Growth-promoting Activity. Serum-free conditioned medium obtained from primary culture of mammary carcinoma cells was assayed for its ability to stimulate DNA synthesis in cultures of quiescent chicken embryo fibroblasts. The results shown in Chart 1 demonstrate that this conditioned medium was very active in the chick cell assay system. As little as 10 μl stimulated [³H]thymidine incorporation significantly, while 100 μl produced a 3.5-fold increase over control levels. It has been shown repeatedly that [³H]thymidine incorporation in this system correlates directly to rates of DNA synthesis (5, 7, 13). The serum-free conditioned medium used in this representative experiment was obtained from primary mammary cell cultures that were incubated in the absence of serum for 96 hr with 2 medium changes during that time period to insure the absence of contaminating serum proteins. It is interesting to note that the cultures survived extremely well under serum-free conditions without decrease in cell number for up to 12 days. Typical “dome” structures were continuously formed and eventually sloughed off into the medium.

Sephadex G-50 Chromatography of Mammary Tumor Cell-conditioned Medium. Since it has been shown that the somatomedins are a significant portion of the serum requirement for the growth of chicken embryo fibroblasts (2), it was of interest to determine whether the activity present in mammary tumor cell-conditioned medium resembled this important family of growth-promoting polypeptides.

In order to obtain adequate quantities of material for these experiments, minced tumor tissue was incubated directly in serum-free medium (see “Materials and Methods” for details of the experiments, minced tumor tissue was incubated directly in serum-free medium (see “Materials and Methods” for details...)
of this procedure). Ten-mg quantities of protein from conditioned medium obtained in this manner were subsequently chromatographed over a 1- x 120-cm Sephadex G-50 column equilibrated in 1 m acetic acid at 4°. Similar procedures have been used for the purification of somatomedins from serum- and BRL-3A rat liver cell-conditioned medium (9, 12, 22). Fractions of 2.5 ml were collected, and aliquots were lyophilized and assayed for protein content and for their ability to stimulate [3H]thymidine incorporation in the chick fibroblast assay (Chart 2). This profile shows that most of the protein eluted in the column void volume while the DNA synthesis-stimulating activity eluted in 2 major peaks, one immediately after the void volume and a second between the elution position of MSA (M.W. 9000) and insulin (M.W. 6000). This peak, which correlates most closely in size to the somatomedins (M.W. ~7500), was chosen for further characterization. The activity in the void volume region was not further characterized. It may represent a distinct growth factor or a higher-molecular-weight form of insulin-like activity. The fractions comprising the lower-molecular-weight region peak were pooled, lyophilized, and resuspended in PBS, pH 7.4. This partially purified preparation from tumor cell-conditioned medium (MTF) was compared to highly purified MSA with regard to its ability to stimulate DNA synthesis in chick embryo fibroblasts. Chart 3 shows typical dose-response curves for the stimulation of [3H]thymidine incorporation by the MTF and MSA. At the highest dose used (20 µg/ml), MTF stimulated about one-half as many cells to enter S phase as did MSA. However, the dose-response curve was still increasing steadily at that concentration. At the corresponding MSA dose (200 ng/ml), which has been shown previously to be the optimal dose, a 12-fold increase in [3H]thymidine incorporation was seen. The MTF preparation was approximately 100- to 150-fold less effective than MSA, on a protein basis, in stimulating DNA synthesis in chick embryo fibroblasts. These values are strikingly similar to those reported for a partially purified somatomedin-like polypeptide preparation produced by a line of human fibrosarcoma cells (3, 21).

Electrophoresis of Mammary Polypeptides (MTF). Since the preparations of MTF used in these experiments were obtained from serum-free medium conditioned by minced tumor tissue rather than from serum-free primary cultures, it was essential to establish that the same polypeptides were produced under both sets of culture conditions. Protein samples from conditioned medium obtained by both methods, along with MSA and insulin for reference, were electrophoresed on 7.5% polyacrylamide gel (4 m:8 m) tube gels (Fig. 1). It is apparent that polypeptides of approximately the same molecular weight were produced under both conditions and

![Chart 2. Sephadex G-50 chromatography of serum-free medium conditioned for 12 hr by minced mammary tumor tissue. Ten mg of protein obtained from acid-dialyzed concentrated conditioned medium were chromatographed over a 1- x 120-cm column of Sephadex G-50 (Fine) equilibrated in 1 m acetic acid at 4°. Aliquots of the 2.5-ml fractions were collected and assayed for protein content (○) and DNA-synthesizing activity (△) using the chick fibroblast assay system as described in Chart 1. The elution positions of column markers are indicated by arrows; M.W.: MSA, 9000; insulin (INS.), 6000.](chart2)

![Chart 3. Dose-response curves of quiescent chick embryo fibroblasts to MSA and MTF. Partially purified tumor cell polypeptides obtained from mammary tumor cell-conditioned medium (see Chart 2, Fractions 24 to 27) and highly purified MSA were added to cultures of quiescent chick embryo fibroblasts to compare their relative potencies in stimulating [3H]thymidine incorporation. O, tumor cell-produced MTF (µg/ml); □, highly purified MSA (µg/ml x 10⁻²). Cultures were assayed 12 hr later for [3H]thymidine incorporation as described in Chart 1.](chart3)

![Fig. 1. Acetic acid:urea (4 m:8 m) polyacrylamide gel electrophoresis. Protein samples from serum-free medium conditioned by minced mammary tumor tissue and primary mammary tumor cell cultures were prepared as described in "Materials and Methods." The samples were applied to 7.5% polyacrylamide tube gels (0.5 x 8.0 cm) in 4 m acetic acid:0.6 m urea and electrophoresed toward the cathode at 1.5 mamp/gel using 4 m acetic acid as electrolyte. MSA and insulin were electrophoresed on identical gels as markers. A, primary culture-conditioned medium (30 µg/50 µl); B, G-50-fractioned tumor tissue-conditioned medium (Chart 2 Fractions 24 to 27, 10 µg) C, MSA (5 µg) D, insulin (2.5 µg).](fig1)
migrated on the gels to a position between that of MSA and insulin. These results correlate well with the results of acidic Sephadex G-50 chromatography, in which the tumor produced polypeptides eluted from the column between the positions of MSA and insulin (Chart 2).

**Competitive Binding Assays.** In light of the apparent similarities between the factor from mammary tumor cell-conditioned medium and MSA in regard to biological activity, acid stability, and size, it was of interest to determine if the MTF initiated its effect on cells through interaction with the same surface receptor as MSA. The results of competitive binding experiments using 125I-labeled MSA as tracer are shown in Chart 4. Unlabeled MSA displaced 50% of the iodinated MSA tracer from surface receptors of chicken embryo fibroblasts at a concentration of 60 ng/ml. This correlates well with the biological activity of purified MSA in Chart 3. The MTF preparation also displaced tracer MSA but at higher concentrations, apparently reflecting its relative purity. EGF binding to mouse embryo fibroblasts was not decreased by MTF at similar concentrations (Chart 4B).

**DISCUSSION**

This report is the first evidence for the production of a somatomedin-like growth factor from a mammary carcinoma. The transplantable tumor used in these studies was derived from a chemical carcinogen-induced neoplastic transformation of epithelial cells in organ culture of intact mouse mammary gland (8, 16). Primary cultures of tumor cells were shown to release into the medium a factor which stimulates DNA synthesis in quiescent chicken embryo fibroblasts. This activity was shown to be acid stable and had a molecular weight of 7000 to 9000. It resembled the somatomedin analog MSA in these properties but more importantly was also able to compete with radioactive MSA for specific binding sites on the surface of chicken embryo fibroblasts. MTF did not compete with EGF for binding to mouse embryo fibroblasts and is therefore different from the sarcoma growth factor produced by murine sarcoma virus-transformed fibroblasts (19, 20).

The MTF appears to be a representative of the MSA or somatomedin family and resembles those factors produced by a human fibrosarcoma cell line in culture (3, 21) which also compete with labeled MSA for cell surface receptors on rat, mouse, or human cells. De Larco and Todaro (3) and Todaro et al. (20) have suggested that endogenous production by tumor cells of growth factors for which they have receptors and are therefore capable of responding provides a constant stimulant for cell growth and a growth advantage over normal cells. Whether this is the mechanism used by the tumor cells described in this report remains obscure. Indeed, the biological significance of the production of somatomedin-like polypeptides by mammary tumor cells remains to be elucidated. It will be critical to determine at what point during tumorigenesis the production of these factors is initiated and whether this correlates with recognizable aspects of neoplastic transformation. The mammary organ culture system from which these tumors were derived should facilitate further experiments designed to answer these questions. In preliminary experiments, we have been unable to detect the production of "somatomedin-like" polypeptides by mammary gland hyperplastic outgrowths, the stage just prior to the onset of neoplasia in this system. In other experiments, which will be the subject of a separate report, we have been able to demonstrate that MSA (200 ng/ml) and the tumor-produced polypeptides (200 to 400 ng/ml) are able to promote the growth and development of normal mammary glands in culture in the complete absence of insulin, which is normally supplied at a concentration of 5 μg/ml.

It is interesting to note a recent report by Ptashne et al. (14) concerning the purification of a MSF from serum which promotes the growth of normal mammary epithelium in culture. MSF was also shown to have somatomedin-like activity and competed with labeled MSA for binding sites on rat liver cell membranes. Displacement of MSA as well as somatomedins A and C from receptors required approximately 10-fold higher...
concentrations than the homologous polypeptide. These results are similar to those presented in this report. Whether MSF and MTF are related will require further investigation. In addition, a growth-stimulating factor derived from solid mouse mammary tumors and cultured tumor cells was described previously by Nair and DeOme (10, 11). While this factor was not fully characterized, it was shown to be of low molecular weight and may also be related to the insulin-like growth factors.

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REFERENCES

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